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Angiotensin II activates nuclear transcription factor-κB through AT_1 and AT_2 receptors[1]

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Angiotensin II activates nuclear transcription factor-κB through AT_1 and AT_2 receptors.

Background. Recent evidence suggests that angiotensin II (Ang II) induces a variety of proinflammatory mediators including chemokines. Nuclear factor-κB (NF-κB) activation plays an important role in Ang II-mediated inflammation. The present study investigated which Ang II receptor subtype is involved in NF-κB activation. We focused particularly on the Ang II subtype 2 (AT_2) receptor because we previously observed that Ang II-induction of the chemokine RANTES in vitro and in vivo is mediated through AT_2 receptors.

Methods. AT_1 or AT_2 receptors were selectively overexpressed in COS7 cells that normally do not express Ang II receptors. In addition, rat glomerular endothelial cells (GER) that express AT_1 and AT_2 receptors and PC12 cells that exclusively exhibit AT_2 receptors were studied also. Ang II-receptor expression was confirmed by Western blots of membrane lysates. NF-κB DNA binding in vitro was detected by electrophoretic shift assays. In addition, in vivo transactivation of a reporter gene construct with κ enhancer coupled to luciferase also was investigated. Expression of the inhibitor of κB alpha (IκB-α) was detected by Western blots.

Results. In AT_1 or AT_2 receptor transfected cells, but not untransfected COS7 cells, 10^{-7} mol/L Ang II induced NF-κB DNA binding in vitro, as detected by electrophoretic shift assays and in vivo transactivation of a reporter gene construct. The AT_2 receptor antagonist PD 123319 but not losartan attenuated Ang II-mediated NF-κB activation in COS7 cells transfected with AT_2 receptors. While Ang II also induced NF-κB activation in PC12 cells, this activation was blocked by PD 123319. Finally, stimulation of GERs with Ang II led to the activation of NF-κB through both subtypes of Ang II receptors. Nuclear extracts from COS7 cells transfected with AT_2 receptors and PC12 cells with NF-κB DNA-binding activity consisted of p50/p65 complexes. There was no difference in subunit composition of nuclear proteins from Ang II-stimulated AT_1 receptor transfected COS7 cells. An artificial peptide (p-Amino-Phe^6-Ang II) with a high affinity for the AT_2 receptor also activated NF-κB. Ang II-induced activation of NF-κB was associated with degradation of IκB-α in all studied cell lines.

Conclusions. Our results clearly demonstrate in various cell lines that Ang II induces NF-κB activation through AT_2 receptors. These data may have important therapeutic consequences, because potential Ang II-mediated proinflammatory renal and cardiovascular effects may not be totally antagonized by the currently increased clinical use of AT_1 receptor antagonists.

There is accumulating evidence that angiotensin II (Ang II) is a pivotal pathophysiological factor in chronic renal disease [1–4]. Besides its well-known hemodynamic actions, Ang II exerts growth stimulatory and profibrogenic effects and even exhibits properties of an inflammatory factor [5–9]. In vascular smooth muscle cells (VSMC) and renal cells, including glomerular endothelial and mesangial cells, Ang II induces chemokines such as monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation, normal T cell expressed and secreted (RANTES) expression [10–13]. Nuclear factor-κB (NF-κB) is a key transcription factor in inflammatory diseases and stimulates, among other genes, the transcription of chemokines [14, 15]. Ruiz-Ortega and associates were the first demonstrating that Ang II could activate NF-κB in cultured mesangial cells and VSMC [11, 16]. Although it is generally assumed that Ang II transduces its inflammatory properties through Ang II subtype 1 (AT_1) receptors and mice that lack AT_{1A} receptors reveal an attenuated immune response [8, 17, 18], there is some evidence that AT_2 receptors may play a role. We found that Ang II infusion into rats stimulates RANTES expression in glomerular endothelial cells [13]. This effect was attenuated by an AT_2 receptor antagonist, but not by losartan [13]. Furthermore, studies in VSMC also demonstrated that AT_2-receptors play a role in Ang II-induced NF-κB activation [16]. However, since studies

[1] **See Editorial by Luft, p. 2272.**

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with selective receptor antagonists could be difficult to interpret and there may be an interaction between AT_1 and AT_2 receptors, for the present study we chose a different approach in selectively overexpressing AT_1 and AT_2 receptors in COS7 cells. Furthermore, a rat glomerular endothelial cell line that expresses AT_1 and AT_2 receptors, and PC12 cells that only bear AT_2 receptors, were studied in parallel. Our results unequivocally demonstrate that Ang II activates NF-κB through AT_1 and AT_2 receptors.

METHODS

Cell culture

PC12 cells, a well-characterized rat adrenal pheochromocytoma cell line, were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Cells were grown in suspension cultures in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Eggenstein, Germany) with 10% fetal calf serum (FCS) in 5% CO_2 at 37°C. In agreement with previous findings [19], PC12 cells expressed only AT_2-receptors by competitive binding assays (data not shown). COS7 cells, originally established from African green monkey kidney, also were obtained from ECACC and grown as monolayers in DMEM with 10% FCS. Glomerular endothelial cells from rat (GERs) are a previously characterized cell line that was established from Wistar rats [20]. These cells express AT_1 and AT_2 receptors [20]. GERs were cultured in DMEM with 10% FCS. A clone of renal tubular LLC-PK_1 cells that expresses only AT_1 receptors [21] was used for some control experiments.

Transient transfections

COS7 cells were transiently transfected with expression vectors in which either the full-length rabbit AT_1 receptor [22] or the rat AT_2 receptor [23] is under control of a cytomegalovirus (CMV) promoter. Transfections were performed in serum-free DMEM with Lipofectin™ (Gibco-BRL). After 24 hours, cells were stimulated with 10^{-7} mol/L Ang II (Sigma, Deisenhofen, Germany) in the presence or absence of either 10^{-6} mol/L losartan (gift of Merck, Sharp, and Dohm, Munich, Germany) or PD123319 (Sigma).

NF-κB transactivation assay

To test whether induced NF-κB does indeed bind and activate target genes, cells were transfected with 10 μg of pNF-κB-Luc, and the same amount of the plasmid pSV-β-galactosidase in which the β-galactosidase gene is under control of the SV40 promoter and enhancer. The pNF-κB-Luc reporter plasmid contains four tandem copies of the κ enhancer fused to the herpes simplex virus thymidine kinase promoter [24]. Activation results in transcription of the luciferase gene. After transfection, the medium was changed and cells were incubated for 12 hours with control medium (serum-free DMEM) or a single dose of 10^{-7} mol/L Ang II. At the end of the experiments, cells were washed three times in PBS, cell layers were lysed, and protein concentrations of supernatants were adjusted to equal concentrations. Luciferase and β-galactosidase activities were measured with standard techniques. A ratio between luciferase and β-galactosidase activities was calculated, and data from unstimulated control cells was assigned an arbitrary value of 1.0. Transient transfections and reporter gene assays were independently performed at least three times for each experiment.

Western blots

For Western blot experiments, cells were stimulated as appropriate. After washing in phosphate buffered saline (PBS), cells were lysed in 2% sodium dodecyl sulfate (SDS), 60 mmol/L Tris-HCl (pH 6.8), 100 mmol/L dithiothreitol (DTT), and the protein content was measured by a modification of the Lowry method that is insensitive to the concentrations of SDS and DTT. Protein concentrations were adjusted to 80 μg/sample, and 5% glycerol, 0.03% bromophenol blue were added and samples were boiled for five minutes. After centrifugation, supernatants were loaded onto a denaturing 10% SDS-polyacrylamide gel. Rainbow markers (Amersham, Braunschweig, Germany) served as the molecular weight standards. After completion of electrophoresis, proteins were electroblotted onto a nitrocellulose membrane in transfer buffer (50 mmol/L Tris-HCl, pH 7.0; 30 mmol/L glycine, 20% methanol, 0.1% SDS). Filters were stained with Ponceau S to control for equal loading and transfer. The blots were blocked in 8% non-fat dry milk in PBS with 0.1% Tween for one hour at room temperature. For the detection of Ang II-receptor expression, specific polyclonal rabbit anti-AT_1 or AT_2 receptor antibodies were used in a 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Lysates of LLC-PK_1 cells, a tubular cell line that exclusively express AT_1-receptors [21], served as an additional control. For the detection of IκB-α, a rabbit polyclonal antibody (New England Biolabs, Beverly, MA, USA) was used in a 1:1000 dilution. The secondary goat anti-rabbit IgG peroxidase–conjugated antibody (BD Bioscience, Heidelberg, Germany) was used in a 1:1000 dilution. The enhanced chemiluminescence system (ECL) system (Amersham) was used for chemiluminescence detection. To detect for small variations in protein loading and transfer, the membranes were washed and re-incubated with a monoclonal antibody against the housekeeping protein β-actin (Sigma). Exposed films were scanned with Fluor-S multi-imager (Bio-Rad Laboratories, Hercules, CA; USA), and data were analyzed with the computer program MultiAnalyst. A ratio between the intensities of IκB-α and β-actin bands was

calculated and cells without Ang II were arbitrarily assigned a relative value of 1.00. Western blotting experiments were independently performed at least three times with qualitatively similar results.

Gel shift assay

To investigate whether NF-κB activation can be induced by Ang II, gel shift assays were performed. Cells were stimulated as appropriate with 10^{-7} mol/L Ang II in the presence or absence of either losartan (10^{-6} mol/L) or the same concentration of PD 123319. Some cells also received p-Amino-Phe^{6}-angiotensin II (Asp-Arg-Val-Tyr-Ile-p-Amino-Phe-Pro-Phe; Sigma), a specific agonist for the AT_2 receptor [25]. After incubation, cells were directly washed twice in flasks with ice-cold PBS and were scraped off the bottom using a rubber policeman. Cells were centrifuged, pellets were transferred to Eppendorf cups, and resuspended in buffer A composed of 20 mmol/L HEPES, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L egtazic acid (EGTA), 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptin, and 1 μg/mL aprotinin. Cells were lysed by the addition of 10% Nonidet-P40 for 10 seconds, lysates were immediately centrifuged for 30 seconds at 13,000 rpm, and pellets were resuspended in buffer B (30 mmol/L HEPES, 0.5 mol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin). After another centrifugation step at 13,000 rpm for five minutes, supernatants containing nuclear proteins were aliquoted, frozen, and stored in liquid nitrogen. At total of 3.50 pmol of double-strand consensus oligonucleotides for NF-κB (5′AGTT GAGGGGACTTTCCCAGGC3′) was end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) using T4 polynucleotide kinase. Binding reactions were performed in gel shift binding buffer [10 mmol/L Tris-HCl, pH 7.5, 4% glycerol, 1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 50 μg/mL poly (dI-dC) poly (dI-dC)] with and without cold competitor oligonucleotides at room temperature for 30 minutes. OCT1 double-strand consensus oligonucleotides (5′TGTCGA ATGCAAATCACTAGAA3′) were used as non-specific competitors. For determination of the subunit composition of DNA-protein complexes, 2 μg of a monoclonal mouse anti-p65/RelA antibody (Boehringer Mannheim, Germany) or of a polyclonal gout anti-p50 antibody (Santa Cruz Biotechnology) was added one hour before incubation with oligonucleotides. Reactions were stopped by addition of loading buffer and samples were run on a non-denaturing 6% polyacrylamide gel. Gels were then exposed to x-ray films. Specific bands were scanned by densitometry as described above and binding of nuclear proteins from cells without Ang II was assigned a value of 1.00. Gel shift analysis was independently performed at least three times (separate cell stimulation, nuclear extract preparation, and binding reactions) with qualitatively similar results.

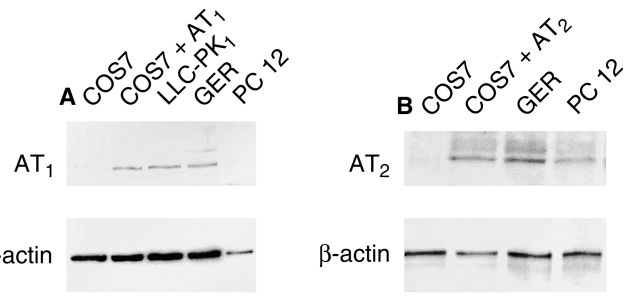

Fig. 1. Detection of angiotensin II subtype 1 (AT_1; *A*) and AT_2 (*B*) receptor expression using Western blots incubated with specific antibodies. (A) A band of approximately 45 kD specific for AT_1 receptor is detected in AT_1 receptor transfected COS7 cells and rat glomerular endothelial cells (GERs). Tubular LLC-PK_1 cells known to express AT_1 receptors served as positive control. Untransfected COS7 cells and PC12 cells did not express AT_1 receptors. (B) AT_2 receptors are detected in GERs, PC12 cells, and COS7 transfected with AT_2 receptor expression plasmid, but not in untransfected COS7 cells. Re-incubation of the blot with an antibody against β-actin revealed that these differences are not due to an uneven transfer of protein. This blot is representative of three independent experiments with qualitatively similar changes.

Statistical analysis

All data are presented as means ± SEM. Statistical significance between different groups was first tested with the non-parametric Kruskal-Wallis test. Individual groups were subsequently tested using the Wilcoxon-Mann-Whitney test. A P value of <0.05 was considered significant.

RESULTS

AT_1, AT_2 receptor expression

Angiotensin II subtype 1 and AT_2 receptor expression in our cells was first examined using Western blots of membrane lysates. A shown in Figure 1A, GERs and COS7 cells transfected with the AT_1 receptor plasmid exhibited AT_1 protein expression, but not untransfected COS7 cells and PC12 cells. Lysates of tubular LLC-PK_1 cells that express only AT_1 receptors served as a positive control. Re-incubation of membranes with an antibody against β-actin revealed that protein was loaded onto each lane (Fig. 1A). On the other hand, GER, PC12 cells, and COS7 cells transfected with the AT_2 receptor plasmid exhibited AT_2 receptor expression, but not untransfected COS7 cells (Fig. 1B).

Reporter construct studies

To test a functional role of Ang II-mediated NF-κB activation on gene transcription, a reporter plasmid containing four tandem copies of the κ enhancer was transfected into the various cell lines. As shown in Table 1, 10^{-7} mol/L Ang II for 24 hours significantly stimulated transactivation of the reporter construct in GER. This

Table 1. Transcriptional activation of pNF-κB-Luc

	Luciferase activity/ β-galactosidase activity
COS7/untransfected controls	1.0 ± 0.0
COS7/untransfected + 10^{-7} mol/L Ang II	0.9 ± 0.4
COS7/AT_1-receptor transfected controls	1.0 ± 0.0
COS7/AT_1-receptor transfected + 10^{-7} mol/L Ang II	1.5 ± 0.2[a]
COS7/AT_2-receptor transfected controls	1.0 ± 0.0
COS7/AT_2-receptor transfected + 10^{-7} mol/L Ang II	1.6 ± 0.1[a]
GER controls	1.0 ± 0.0
GER + 10 ng/mL TNF-α	1.6 ± 0.1[a]
GER + 10^{-7} mol/L Ang II	2.4 ± 0.2[a]
GER + Ang II + 10^{-6} mol/L Los	1.6 ± 0.2[c]
GER + Ang II + 10^{-6} mol/L PD 123319	1.5 ± 0.2[c]
GER + Ang II + Los + PD 123319	1.1 ± 0.1
PC12 controls	1.0 ± 0.0
PC12 + 10^{-7} mol/L Ang II	1.5 ± 0.1[b]
PC12 + Ang II + 10^{-6} mol/L Los	1.6 ± 0.2[a]
PC12 + Ang II + 10^{-6} mol/L PD 123319	0.7 ± 0.2[c]

$N = 3$–6.
[a] $P < 0.05$ versus unstimulated controls
[b] $P < 0.01$ versus unstimulated controls
[c] $P < 0.05$ versus Ang II only

effect was partly blocked by either losartan or PD 123319 suggesting involvement of both AT_1 and AT_2 receptors. A combination of losartan and PD 123319 almost completely blocked Ang II-induced transcription of the pNF-κB-Luc reporter plasmid (Table 1). COS7 cells transfected with the AT_1 or AT_2 receptor expression constructs, but not mock-transfected cells, revealed transactivation of NF-κB in the presence of Ang II (Table 1). Finally, Ang II significantly induced gene transcription by stimulating the κ enhancer in PC12 cells that exclusively expressed AT_2 receptors (Table 1). This transcriptional activity was attenuated by PD 123319 but not by losartan (Table 1).

NF-κB binding in vitro detected with EMSAs

The kinetic analysis of NF-κB DNA-binding activity in vitro in various types of cells is demonstrated in Figure 2. In AT_1 and AT_2 receptor transfected COS7 cells, a single dose of 10^{-7} mol/L Ang II induced binding of nuclear proteins to consensus oligonucleotides after 30 to 60 minutes (Fig. 2B), but not in untransfected COS7 cells (Fig. 2A). Even longer exposure of untransfected COS7 cells with Ang II for 120 minutes failed to induce a gel shift (Fig. 2A). However, Ang II also induced NF-κB activation in GERs and PC12 cells (Fig. 2 B, C).

As shown in Figure 3A, Ang II-mediated NF-κB binding in AT_1 receptor transfected COS7 cells is attenuated by 10^{-6} mol/L losartan, but not by the same concentration of PD 123319. In contrast, 10^{-6} mol/L PD 123319 attenuated NF-κB binding in COS7 cells transfected with the AT_2 receptor expression plasmid (Fig. 3A). Both losartan as well as PD 123319 diminished NF-κB activation in GERs (Fig. 3B). A combination of AT_1 and AT_2 receptor antagonists was more effective than each drug alone (Fig. 3B). Finally, PD 123319 but not losartan inhibited Ang II-mediated NF-κB activation in PC12 cells (Fig. 3C).

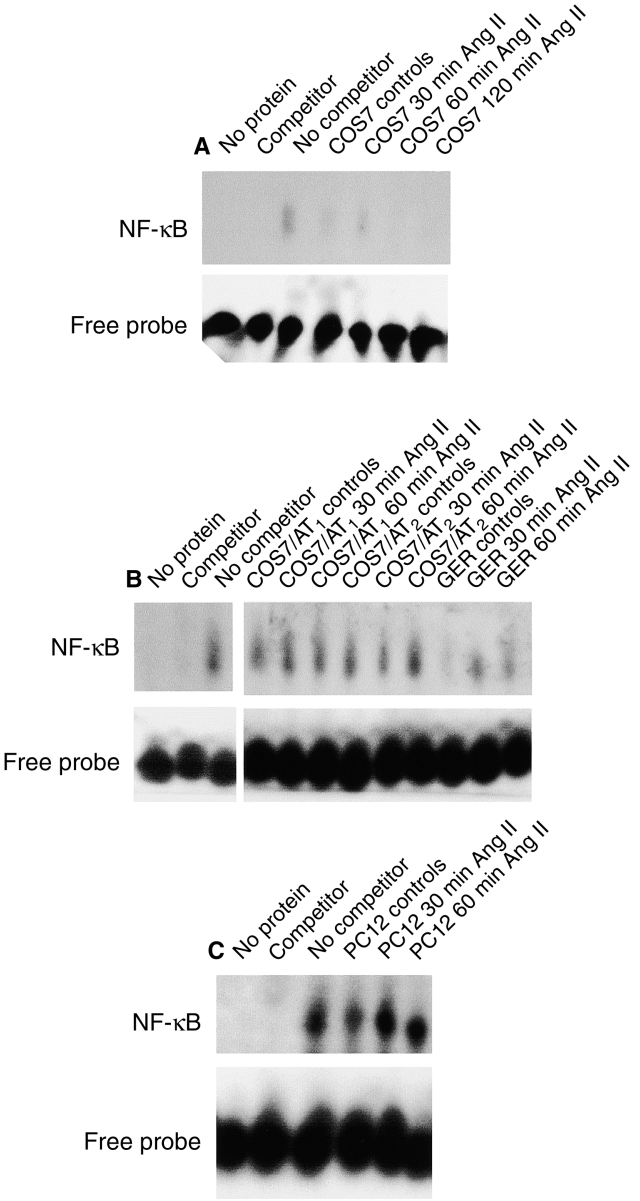

Fig. 2. Nuclear factor-κB (NF-κB) binding in vitro as detected with electrophoretic mobility shift assays (EMSAs). (*A*) Non-transfected COS7 cells showed no increase in NF-κB binding to consensus oligonucleotides after stimulation with 10^{-7} mol/L angiotensin II (Ang II). (*B*) In contrast, COS7 cells either transfected with AT_1 or AT_2 receptor expression constructs revealed in increase in NF-κB binding as early as 30 minutes after stimulation with Ang II (10^{-7} mol/L). In addition, GERs treated with Ang II exhibit an increase in NF-κB binding to oligonucleotides. (*C*) Challenge of PC12 cells with 10^{-7} mol/L Ang II also induced NF-κB activation. The specificity of the reactions was established using nuclear proteins from either Ang II-treated AT_1 receptor transfected COS7 cells (A and B) or Ang II-stimulated PC12 cells (C) with a 100-fold excess of unlabeled NF-κB (competitor) or unrelated OCT1 oligonucleotides (non-competitor). A densitometric analysis of experiments is shown in Table 2.

To gain insight into subunit composition of NF-κB

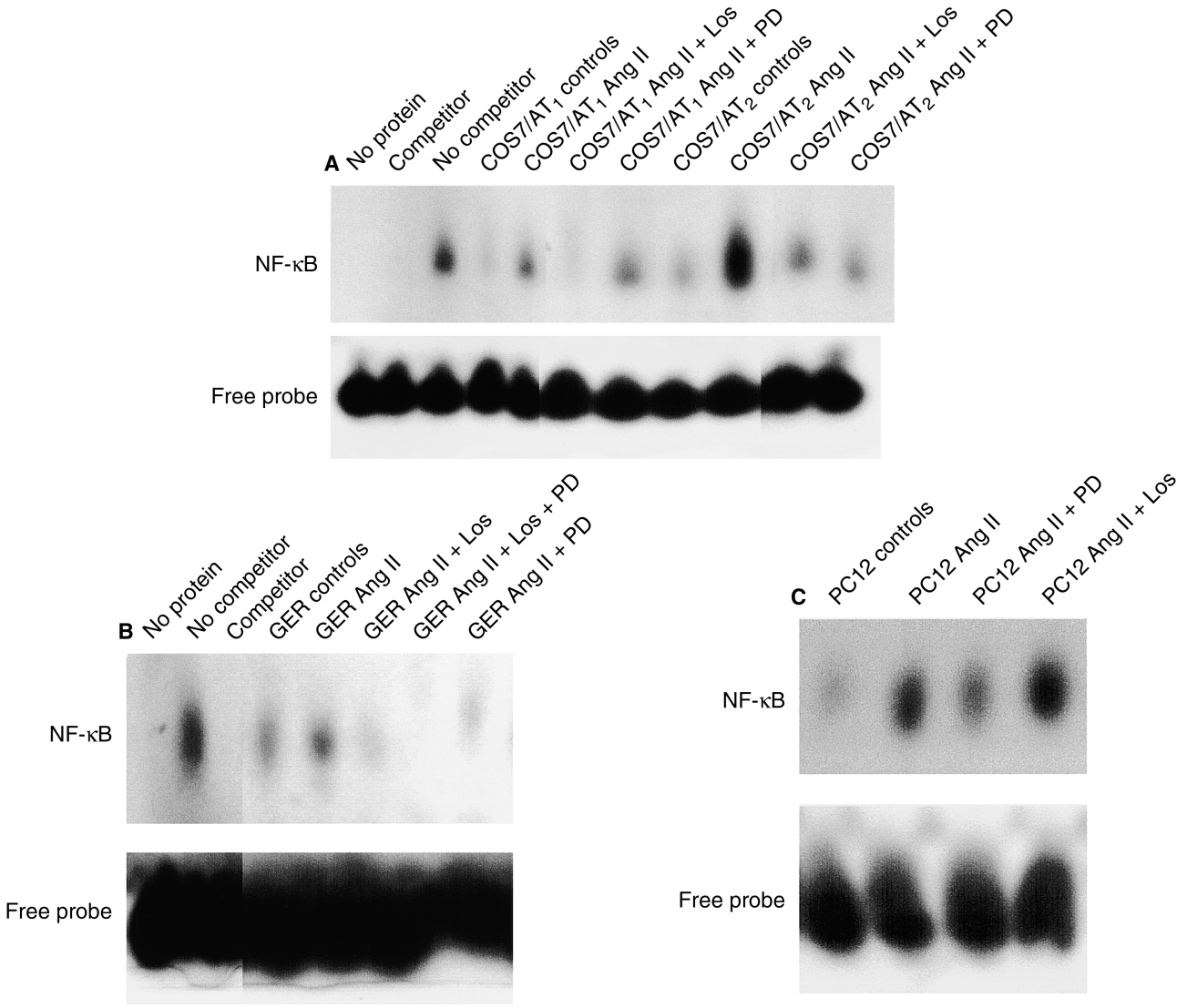

Fig. 3. Effect of AT_1 and AT_2 receptor antagonists on NF-κB binding in various cell lines. (*A*) Treatment of COS7 cells transfected with the AT_1 receptor expression plasmid with 10^{-6} mol/L losartan (Los) almost completely attenuated NF-κB binding induced by 10^{-7} mol/L Ang II. In contrast, 10^{-6} mol/L PD 123319 (PD) was without significant effect. Although 10^{-6} mol/L losartan somewhat reduced Ang II-mediated NF-κB activation in COS7 cells transfected with AT_2 receptors, the same concentration of PD 123319 was more effective. (*B*) The AT_1 receptor antagonist losartan (10^{-6} mol/L Los) as well as the AT_2 receptor blocker (10^{-6} mol/L PD) attenuated Ang II-induced NF-κB binding to oligonucleotides in nuclear protein preparations obtained from GERs. A combination of losartan and PD 123319 was more effective than each drug alone. (*C*) Only 10^{-6} mol/L PD 123319 (PD) attenuated NF-κB binding in Ang II-treated PC12 cells whereas losartan (10^{-6} mol/L Los) was without effect. Specificity of the reactions was confirmed with a 100-fold excess of unlabeled NF-κB (competitor) or unrelated OCT1 oligonucleotides (non-competitor). A densitometric analysis of experiments is shown in Table 2.

protein-DNA complexes, supershift experiments were performed. Nuclear proteins prepared from Ang II-treated PC12 cells and AT_2 receptor transfected COS7 cells revealed a supershift of complexes in the presence of anti-p50 or anti-p65/RelA antibodies (Fig. 4). In accordance with previous observations [26], supershifts were incomplete possible because of steric hindrance. Supershift experiments with nuclear extracts prepared from AT_1 receptor transfected COS7 cells revealed exactly the some pattern indicating no major difference in NF-κB subunit composition (Fig. 4). Finally, p50 and p65/RelA subunits were also part of the NF-κB obtained from Ang II-treated GERs in which the peptide activates AT_1 and AT_2 receptors (Fig. 4).

p-Amino-Phe^6-angiotensin II (pNH_2F-Ang II), which preferentially binds to AT_2 receptors, was tested in additional studies. As demonstrated in Figure 5, 10^{-7} mol/L pNH_2F-Ang II for 30 minutes induced NF-κB activation in GERs, PC12 cells, and COS7 cells transfected with the AT_2 receptor expression plasmid.

A densitometric analysis and statistical significance of EMSAs is provided in Table 2.

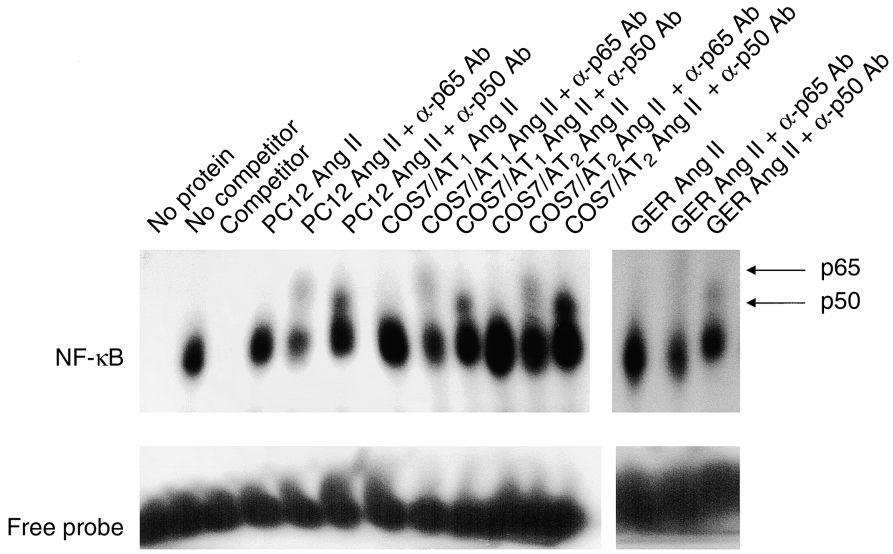

Fig. 4. Supershift experiments to determine subunit composition of NF-κB. Nuclear proteins obtained from AT_1 or AT_2 receptor transfected COS7 cells, PC12 cells, and GERs after stimulation with Ang II were incubated with antibodies against p50 or p65 subunits before addition of radiolabeled oligonucleotides. Gel retardation ("supershift") in the presence of antibodies reveals that this subunit is part of NF-κB. There was not qualitative difference in the supershifts of nuclear proteins isolated from the various cell lines. This indicates that both the p50 and p65 subunits are part of all NF-κB complexes. This EMSA is representative of two independent experiments with qualitatively similar results.

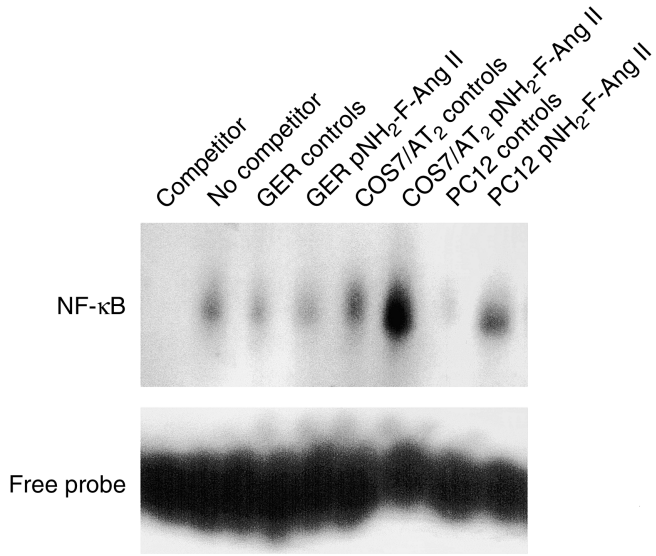

Fig. 5. p-Amino-Phe^6-angiotensin II (pNH_2F-Ang II) induces NF-κB activation. COS7 cells transfected with AT_2 receptors, GERs, and PC12 cells were treated with 10^{-7} mol/L (pNH_2F-Ang II), a peptide with a high affinity for AT_2 receptors. pNH_2F-Ang II activates NF-κB in all three cell lines. This blot is representative of three independent experiments with qualitatively similar changes.

Table 2. Densitometric quantification of nuclear factor-κB (NF-κB) activation (EMSAs)

	Arbitrary units (controls = 1.00)
A. COS7 cells untransfected	
Controls	1.00 ± 0.00
30 minutes Ang II	1.02 ± 0.02
60 minutes Ang II	0.98 ± 0.10
120 minutes Ang II	1.11 ± 0.03
B. COS7 cells AT_1 receptor transfected	
Controls	1.00 ± 0.0
30 minutes Ang II	2.43 ± 0.08[b]
60 minutes Ang II	1.23 ± 0.06[b]
60 minutes Ang II + losartan	1.02 ± 0.20[c]
60 minutes Ang II + PD 123319	2.51 ± 0.80[a]
C. COS7 cells AT_2 receptor transfected	
Controls	1.00 + 0.00
30 minutes Ang II	2.49 ± 0.30[b]
60 minutes Ang II	4.24 ± 1.02[a]
60 minutes Ang II + losartan	2.94 ± 0.12[a]
60 minutes Ang II + PD 123319	1.18 ± 0.06[c]
D. GERs	
Controls	1.00 ± 0.00
30 minutes Ang II	1.27 ± 0.15[a]
60 minutes Ang II	1.30 + 0.10[a]
60 minutes Ang II + losartan	1.20 ± 0.08
60 minutes Ang II + PD 123319	1.12 ± 0.10
60 minutes Ang II + losartan + PD 123319	1.01 + 0.03[c]
E. PC12 cells	
Controls	1.00 ± 0.00
30 minutes Ang II	1.35 ± 0.07[a]
60 minutes Ang II	1.31 + 0.04[a]
60 minutes Ang II + losartan	2.00 ± 0.11[a]
60 minutes Ang II + PD 123319	1.10 ± 0.05[c]

N = 4–8.
[a] $P < 0.05$ versus unstimulated controls
[b] $P < 0.01$ versus unstimulated controls
[c] $P < 0.05$ versus cells treated with Ang II only

Ang II-mediated degradation of IκB-α

To further investigate potential molecular mechanisms for Ang II-mediated activation of NF-κB, we studied IκB-α expression using Western blots. Figure 6 shows IκB-α expression in AT_1 receptor-transfected COS7 cells (A), AT_2 receptor-transfected COS7 cells (B), GERs (C), and PC12 cells (D) 30 to 120 minutes after treatment with 10^{-7} mol/L Ang II. The membranes were reincubated with an anti-β-actin antibody to control for loading and transfer of proteins. Ang II treatment reduces IκB-α expression after 30 to 60 minutes. Densitometric analysis of independent experiments is given in Table 3.

DISCUSSION

Accumulating evidence suggests that Ang II exerts proinflammatory properties in various tissues including vessels and kidney [5–8, 27]. Treatment of immune and non-immune mediated models of chronic nephropathy

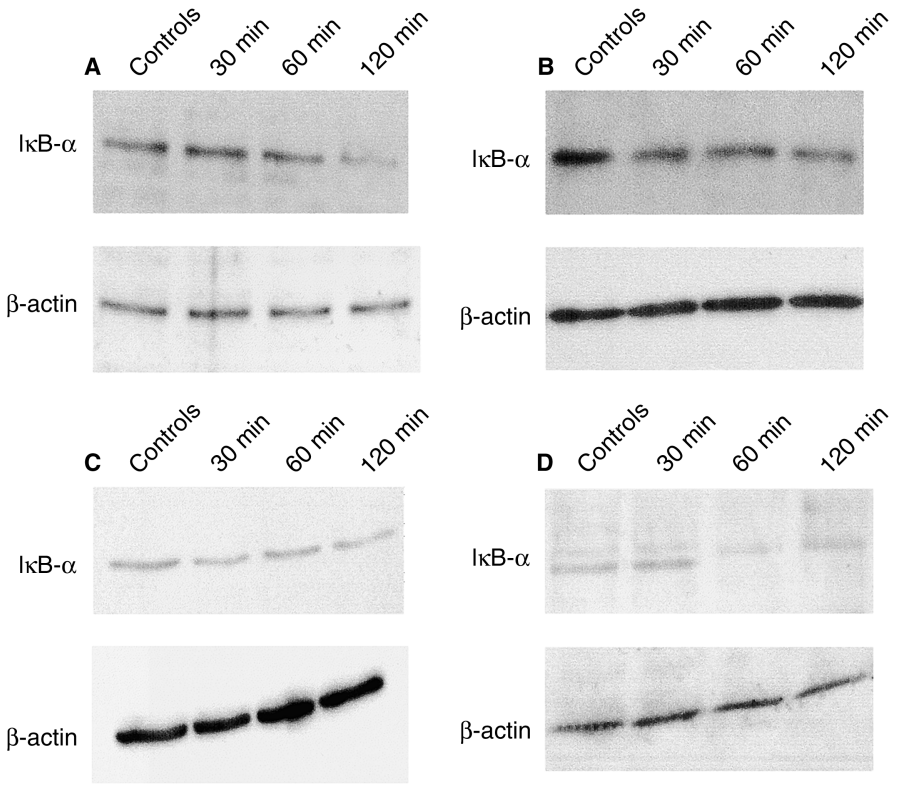

Fig. 6. Western blot for IκB-α expression in AT_1 receptor-transfected COS7 cells (*A*), AT_2 receptor-transfected COS7 cells (*B*), GERs (*C*), and PC12 cells (*D*) 30 to 120 minutes after treatment with 10^{-7} mol/L Ang II. The membranes were re-incubated with an anti-β-actin antibody to control for loading and transfer of proteins. Ang II treatment reduces IκB-α expression after 30 to 60 minutes. Densitometric analysis of 3 to 4 independent experiments is given in Table 3.

Table 3. Densitometric quantification of IκB-α expression

	Relative IκB-α expression normalized to β-actin
A. COS7 cells AT_1 receptor transfected	
Controls	1.00 ± 0.00
30 minutes Ang II	0.95 ± 0.09
60 minutes Ang II	0.75 ± 0.07[a]
120 minutes Ang II	0.41 ± 0.15[a]
B. COS7 cells AT_2 receptor transfected	
Controls	1.00 ± 0.00
30 minutes Ang II	0.81 ± 0.09[a]
60 minutes Ang II	0.75 ± 0.12[a]
120 minutes Ang II	0.62 ± 0.13[b]
C. GERs	
Controls	1.00 ± 0.00
30 minutes Ang II	0.65 ± 0.12[a]
60 minutes Ang II	0.67 ± 0.06[b]
120 minutes Ang II	0.61 ± 0.04[b]
D. PC12 cells	
Controls	1.00 ± 0.00
30 minutes Ang II	0.83 ± 0.10[a]
60 minutes Ang II	0.51 ± 0.06[b]
120 minutes Ang II	0.84 ± 0.08[a]

$N = 3$ for GERs, $N = 4$ for PC12 cells, $N = 5$ for COS7 cells transfected with either AT_1 or AT_2 receptors.
[a] $P < 0.05$ versus unstimulated controls
[b] $P < 0.01$ versus unstimulated controls

with angiotensin-converting enzyme (ACE) inhibitors reduces glomerular and tubulointerstitial macrophage/monocyte infiltration [27–32]. Ang II has chemoattractant properties through local induction of chemokines. For example, in cultured renal endothelial and mesangial cells, Ang II induces chemokines such as MCP-1 and RANTES [10, 13]. Renal MCP-1 expression is partly inhibited by AT_1-receptor antagonists in mesangioproliferative glomerulonephritis, diabetic nephropathy, unilateral ureteral obstruction, and chronic transplant rejection [28, 30, 33, 34].

Nuclear factor-κB is an important regulator of immune responses by mediating transcription of chemokines including MCP-1 and RANTES. Although initially thought to be B cell specific [35], NF-κB activity can be induced in many cells by a wide range of different factors. NF-κB exists in the cytoplasm in an inactive form associated with IκB-α. After phosphorylation, IκB-α is degraded and NF-κB translocates into the nucleus [36].

Several in vitro and in vivo studies provide ample evidence that Ang II is a key mediator of NF-κB activation [6, 9, 11, 31, 37]. Egido's group was among the first to show in elegant in vivo studies that ACE inhibitor treatment reduces NF-κB expression and macrophage/monocyte infiltration in a rabbit model of atherosclerosis, in immune complex nephritis, and, more recently, in rats with overload proteinuria [6, 27, 31, 38]. Morrissey and Klahr demonstrated that enalapril decreases NF-κB in the kidney with unilateral ureteral obstruction [39]. Increased urinary protein excretion also increased NF-κB activation in rats after 5/6 nephrectomy and passive Heymann nephritis [33]. This NF-κB activation was paral-

leled by an up-regulation of MCP-1 transcripts and was inhibited by the ACE inhibitor lisinopril [33]. Although the inhibitory effects of ACE inhibitor on NF-κB activation in the proteinuric models may be partly explained by a reduction in proteinuria, there is also evidence that Ang II directly activates NF-κB [33]. Müller and associates found that NF-κB is a key mediator of inflammation in transgenic rats harboring both human renin and angiotensinogen genes [9]. Systemic infusion of Ang II into naïve rats activates NF-κB in the kidney [40]. Interestingly, an AT_1 receptor antagonist diminished NF-κB activity in glomerular and tubular cells, whereas an AT_2 receptor blocker attenuated only glomerular macrophages/monocyte infiltration and NF-κB activation, suggesting that both subtypes of Ang II receptors are involved in NF-κB activation [40].

Although there are convincing data that Ang II activates NF-κB in renal and cardiovascular cells, the subtype of Ang II receptor that is involved in this proinflammatory process remains incompletely understood. Ang II binds to at least two different types of receptors, AT_1 and AT_2 [41–44]. AT_1 receptors are responsible for mediating many of the well-known stimulatory actions of Ang II on vasoconstriction, sodium intake, aldosterone secretion, cell growth, and fibrogenesis [41]. In contrast, the function of AT_2 receptors is less clear. However, recent evidence suggests that these receptors mediate antiproliferative effects, apoptosis, and release of nitric oxide [43, 44]. As a simplification, it has been assumed that AT_2 receptor activation leads to principally opposite effects compared to binding of Ang II to AT_1 receptors, and a "Yin-Yang" hypothesis of AT_1/AT_2 receptors has been proposed [43, 44]. Therefore, we were surprised to discover that Ang II-stimulated induction of RANTES in vitro and in vivo is attenuated by an AT_2 receptor antagonist but not by losartan [13]. Moreover, although losartan lowered blood pressure induced by Ang II-infusion into naive rats, it failed to influence glomerular infiltration of macrophages/monocytes [13]. In contrast, in vivo application of PD 123319 significantly attenuated glomerular macrophage/monocyte influx without influence of blood pressure [13]. These data clearly suggest that proinflammatory effects of Ang II can be transduced by AT_2 receptors [13].

The present study provides clear evidence that binding of Ang II to AT_2 receptors leads to activation of the proinflammatory factor NF-κB. Ang II stimulates NF-κB activation in PC12 cells that only express AT_2 receptors. Furthermore, PD 123319 partly attenuates NF-κB activation in GERs that exhibit both AT_1 and AT_2 receptors. Untransfected COS7 cells did not express Ang II receptors and the peptide consequently failed to induce NF-κB. However, Ang II activates NF-κB in COS7 cells transfected with an AT_2 receptor expression construct. This activation was antagonized by an AT_2 receptor antagonist. Finally, the peptide pNH_2F-Ang II with a high affinity to AT_2 receptors activates NF-κB in PC12 cells, GERs, and COS7 cells transfected with AT_2 receptors. In accordance with previous studies [11, 16], Ang II also induces NF-κB through AT_1 receptors in GERs and COS7 cells transfected with AT_1 receptor expression plasmids. Limited supershift experiments provided evidence that NF-κB complexes are composed of p50 and p65 (RelA), the most common of the subunits. In accordance with previous studies [26], supershift of bands was incomplete suggesting steric hindrance. Since we have not performed supershift experiments for all possible subunits, we could not totally rule out that other subunits may have a minor contribution to formation of NF-κB activity. However, there was no difference in composition of subunits in regard to AT_1 or AT_2 receptor activation.

Degradation of IκB-α was associated with NF-κB activation, strongly suggesting that Ang II induces phosphorylation and ubiquination of this inhibitory protein. Previous in vivo studies in Ang II-infused rats as well as cell culture investigations with VSMC using specific antagonists provided evidence that Ang II activates NF-κB through both the AT_1 and AT_2 receptors [16, 40]. However, relying only on receptor antagonists may be problematic because these agents are not totally specific. Furthermore, there is cross-talking between AT_1 and AT_2 receptors, and binding of Ang II to one subtype of receptor may modulate expression of the other type [44]. Particular AT_2 receptors may be up-regulated in injured tissue making an interpretation of findings difficult [43, 44].

We therefore believe that our current approach with selective reconstitution of AT_1 or AT_2 receptors provides compelling evidence that Ang II can indeed activate NF-κB through both receptor types. In this regard, the ability of the AT_2 receptor to activate NF-κB is important. Recent studies of Ruiz-Ortega and coworkers found additional evidence in VSMC obtained from AT_1 receptor-deficient mice that Ang II activates NF-κB in those cells that only express AT_2 receptors [45]. Angiotensin III also activates NF-κB in mesangial cells obtained from AT_1 knockout mice, further underscoring the ability of this receptor to engage with proinflammatory signal transduction pathways [46]. AT_1 and AT_2 receptors interact with different signal transduction intermediates [41–43]. Among other effectors, tyrosine kinase activation has been described as a principal consequence of AT_1 receptor activation whereas Ang II-binding to the AT_2 receptor subtype involves modification of phosphatase activity [41–43]. It will be an important subject of future studies to identify potential signal transduction mechanisms leading to NF-κB activation through AT_1 or AT_2 receptors.

In vitro binding studies of NF-κB may not necessarily reflect transcriptional activation of target genes by NF-κB. Investigating proinflammatory genes such as MCP-1 and RANTES may not be conclusive, because Ang II-medi-

ated transcription of these genes could involve other transcription factors such as activator protein-1 (AP-1) [40]. Consequently, we and others used a reporter gene construct in which an enhancer with multiple κ binding sites drives the luciferase gene [16]. We provide compelling evidence that Ang II-induced NF-κB indeed *trans*-activates a reporter gene in vivo through either AT_1 or AT_2 receptors.

Our finding that Ang II can activate NF-κB through binding to either AT_1 or AT_2 receptors may have clinical consequences. Clinical studies investigating AT_1 receptor antagonists in diabetic renal disease clearly show that these drugs could prevent progression of renal disease [47, 48]. In addition, several experimental investigations also clearly demonstrated that AT_1 receptor treatment exerts anti-inflammatory effects [7, 10, 16, 17]. Renal NF-κB activation is significantly attenuated in AT_1 receptor deficient mice with protein overload nephropathy [49]. Nevertheless, currently it is unclear whether ACE inhibitors are equal, better or even worse than AT_1 receptor antagonists. Some studies in the cardiovascular field revealed that ACE inhibitors may be better that AT_1 receptor antagonists [50–52]. Yet the future probably lies with a combination of ACE inhibitor and AT_1 receptor blocker therapy [53, 54]. Based on our in vitro studies, one can speculate that AT_1 receptor antagonists may not be as effective as ACE inhibitors to antagonize Ang II-mediated proinflammatory effects induced by NF-κB. However, whether AT_2 receptor-induced NF-κB activation plays any role in clinical situations in the presence of AT_1 receptor blockade remains unclear. Further studies are necessary to unravel whether AT_2 receptor-mediated NF-κB activation occurs in human renal disease.

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